REMARKS

This application has been amended in a manner that is believed to place it in condition for allowance at the time of the next Official Action.

Claims 1-2 and 4-22 are pending in the application. Claims 1-2 and 4-12 have been amended to address formal matters. New claims 13-22 have been added. Support for claims 13-22 may be found in the original claims and generally throughout the specification.

In the outstanding Official Action, the specification was objected to for allegedly containing trademarks. Pursuant to the Examiner's suggestion, the specification has been amended so that trademarks are capitalized wherever they appear in the specification. In regards to the generic terminology, it is believed that the trademarks that are present are already sufficiently described. For example, the specification indicates on page 3 that the recited trademarks are fluorescent molecules. Thus, it is respectfully requested that the objection be withdrawn.

Claims 3, 8 and 10 were objected to for allegedly containing several informalities. Claims 3, 8 and 10 have been amended to address these informalities. Applicants thank the Examiner for his suggestions as how to overcome the objections in the specification and claims.

Claims 1-6 and 8-11 were rejected under 35 USC 112, second paragraph, for allegedly omitting essential steps. This rejection is respectfully traversed.

Applicants believe that claims 1-6 and 8-11 already satisfied the requirements of 35 USC 112, second paragraph. Nevertheless, in the interest of advancing prosecution, the claims have been amended to recite a step which relates back to the preamble.

The claims have also been amended to recite active steps.

Claim 3 has been cancelled.

Claims 1-6 and 8-11 were rejected under 35 USC 112, first paragraph, for allegedly not satisfying the written description requirement. This rejection is respectfully traversed.

The Examiner is respectfully reminded that the inquiry into whether the description requirement is met must be determined on a case-by-case basis and is a question of fact. *In re Wertheim*, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1976). Moreover, a description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption. See, e.g., *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). The examiner, therefore, has the initial burden of presenting by a preponderance of evidence why a person skilled in

the art would not recognize in an applicant's disclosure a description of the invention defined by the claims. *In re Wertheim*, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1976).

As the Official Action fails to provide any evidence or reason as to why the claimed invention does not support recitations to pre-cancerous lesions, it is respectfully submitted that the Patent Office fails to satisfy its burden in showing that the written description requirement is not satisfied.

Nevertheless, applicants note that dependent claims 21 and 22 have been added which recites that the method is a method for determining a presence of colorectal tumors, and wherein a total amount of amplicons higher than the reference value is indicative of the presence of colorectal tumors in said patient.

Thus, in view of the above, applicants respectfully request that the rejection be withdrawn. Moreover, applicants note that claim 21 particularly satisfies the written description requirement.

Claims 1-2, 4-6, and 9-11 were rejected under 35 USC 102(b) as allegedly being anticipated by SHUBER. This rejection is respectfully traversed.

The method for disease detection/screening described by SHUBER is based on PCR amplification, gel electrophoresis analysis and ethidium bromide staining (see Example 1, page 20). However, SHUBER fails to disclose or suggest the method that

utilizes fluorescent molecules as detectable markers. Accordingly, SHUBER fails to anticipate the claimed invention.

In view of the above, applicants respectfully request that the SHUBER rejection be withdrawn.

Claim 3 was rejected under 35 USC 103(a) as allegedly being unpatentable over SHUBER and further in view of TIAN. Claim 8 was rejected under 35 USC 103(a) as allegedly being unpatentable over SHUBER in view of either KMIEC or in view of ALBERTSEN and BUCK. These rejections are respectfully traversed.

It is respectfully submitted that the publications of TIAN, KMIEC, ALBERTSEN, and BUCK fail to remedy the deficiencies of SHUBER for reference purposes.

SHUBER discloses a method for disease detection/screening based on PCR amplification, gel electrophoresis analysis and ethidium bromide staining (Example 1, page 20). When applied to the diagnosis of colorectal tumors, however, ethidium bromide staining and subsequent quantification allow for a poor discrimination between healthy individuals and colorectal cancer patients. In fact, gel staining does not permit an accurate quantification of PCR amplificons and allows to discriminate only among four groups: null, low, medium or high levels of PCR amplifications (see SHUBER's article published in Gastroenterology 119:1219-1227, 2000, abstract enclosed). In this way, all colorectal cancer patients with fecal DNA levels slightly higher than healthy individuals fall into the same class

or group of healthy individuals with a consequent incorrect attribution of a vast number of colorectal tumors and a low method sensitivity.

Compared to the methods used by SHUBER, the improvement given by the claimed method is significant. As discussed in the enclosed paper, which was published in Neoplasia and authored *inter alios* by the current inventors, the "fluorescent method" according to the invention secures 76% sensitivity and 93% specificity at 25 ng cut-off (see Table 2), whereas the "ethidium bromide method" taught by SHUBER secures 70% sensitivity and 79% specificity (Table 1). The skilled person would not expect that using fluorescent labels to quantitate PCR-amplified DNA from stool samples would determine such a specificity/sensitivity improvement in the screening for colorectal tumors.

Furthermore, the specific sequences which are targeted by the method of the invention (p53 or APC genes) provide an additional inventive contribution to the claimed subject matter. In fact, only 44% sensitivity was recently reported for a quantitative method used to evaluate fecal DNA levels using *Alu* sequence (Cancer Epid Biom Prev, 15:1115-1119, 2006, encl. 5), whereas the sensitivity obtained with the claimed method, which is based on the amplification of p53 and APC gene fragments, results significantly higher (76%, see enclosed paper). At the time of the invention, there was no hint at selecting the claimed sequences as PCR targets in order to improve the sensitivity of a

method for colorectal tumor detection based on quantitative analysis of DNA from a stool sample.

None of the publications of TIAN, KMIEC, ALBERTSEN, or BUCK disclose or suggest a method for detecting the presence of colorectal tumors, wherein the total amount of amplicons higher than the reference value is indicative of the presence of a colorectal tumor in a subject.

Thus, in view of the above, applicants respectfully request that the obviousness rejections be withdrawn.

At this time, applicants respectfully request an action on the merits for all of the claims in their full scope. Applicants maintain that the lack of unity determination is improper for the same reasons outline in the response of January 22, 2007 and again ask that the Examiner withdraw the requirement.

In view of the present amendment and foregoing remarks, therefore, applicants believe that the present application is in condition for allowance at the time of the next Official action. Allowance and passage to issue on that basis is respectfully requested.

Please charge the fee of $25 for one extra dependent claim added herewith to Deposit Account No. 25-0120.

method for colorectal tumor detection based on quantitative analysis of DNA from a stool sample.

None of the publications of TIAN, KMIEC, ALBERTSEN, or BUCK disclose or suggest a method for detecting the presence of colorectal tumors, wherein the total amount of amplicons higher than the reference value is indicative of the presence of a colorectal tumor in a subject.

Thus, in view of the above, applicants respectfully request that the obviousness rejections be withdrawn.

At this time, applicants respectfully request an action on the merits for all of the claims in their full scope. Applicants maintain that the lack of unity determination is improper for the same reasons outline in the response of January 22, 2007 and again ask that the Examiner withdraw the requirement.

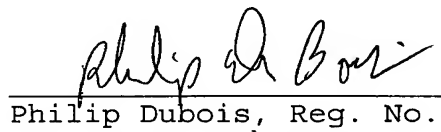
In view of the present amendment and foregoing remarks, therefore, applicants believe that the present application is in condition for allowance at the time of the next Official action. Allowance and passage to issue on that basis is respectfully requested.

Please charge the fee of $25 for one extra dependent claim added herewith to Deposit Account No. 25-0120.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

YOUNG & THOMPSON



Philip Dubois, Reg. No. 50,696
745 South 23rd Street
Arlington, VA 22202
Telephone (703) 521-2297
Telefax (703) 685-0573
(703) 979-4709

PD/lrs

APPENDIX:

The Appendix includes the following items:

- copy of article by CALISTRI et al., "Detection of Colorectal Cancer by a Quantitative Fluorescence Determination of DNA Amplification in Stool", Neoplasia, Vol. 6, No. 5, October, 2004, pp. 536-540;

- copy of article by ZOU et al., "A Sensitive Method to Quantify Human Long DNA in Stool: Relevance to Colorectal Cancer Screening", Cancer Epidemiol. Biomarkers, Prev. 2006; 15(6), June 2006;

- copy of article by AHLQUIST et al., "Colorectal Cancer Screening by Detection of Altered Human DNA in Stool: Feasibility of a Multitarget Assay Panel", www..gastrojournal.org/article/PIIS0016508500895268/abstract?browse_volum...

RESEARCH ARTICLE

Neoplasia • Vol. 6, No. 5, September/October 2004, pp. 536–540 536

www.neoplasia.com

Detection of Colorectal Cancer by a Quantitative Fluorescence Determination of DNA Amplification in Stool[1]

Daniele Calistri[*], *Claudia Rengucci*[*], *Arturo Lattuneddu*[†], *Gianfranco Francioni*[‡], *Anna Maria Polifemo*[§], *Oriana Nanni*[*], *Luca Saragoni*[*], *Franco Monti*[¶], *Alberto Ravaioli*[¶], *Wainer Zoli*[*] *and Dino Amadori*[*]

[*]Division of Oncology and Diagnostics, Pierantoni Hospital, Forlì, Italy; [†]Department of Surgery I, Morgagni Hospital, Forlì, Italy; [‡]Department of General Surgery, Infermi Hospital, Rimini, Italy; [§]Gastroenterology Unit, Morgagni Hospital, Forlì, Italy; [¶]Department of Oncology, Infermi Hospital, Rimini, Italy

Abstract

DNA amplification of exfoliated cells in stool represents an inexpensive and rapid test, but has only 50% to 60% sensitivity. A new quantitative method, called fluorescence long DNA, was developed and validated in our laboratory on stool obtained from 86 patients with primary colorectal cancer and from 62 healthy individuals. It consists of the amplification of stool DNA with fluorescence primers and the quantification of the amplification using a standard curve. Results are arbitrarily expressed in nanograms. The potential of the new method compared to the conventional approach was analyzed in a subgroup of 94 individuals (56 patients and 38 healthy volunteers). In the present series, DNA amplification analysis showed a specificity of 97% and a sensitivity of only 50%. Conversely, fluorescence DNA evaluation, using the best cutoff of 25 ng, showed a sensitivity of about 76% and a specificity of 93%. Similar sensitivity was observed regardless of Dukes stage, tumor location, and size, thus also permitting the detection of early-stage tumors. The present study seems to indicate that quantitative fluorescence DNA determination in stool successfully identifies colorectal cancer patients with a sensitivity comparable, if not superior, to that of multiple gene analysis but at a lower cost and in a shorter time.

Neoplasia (2004) 6, 536–540

Keywords: Colorectal cancer, stool, DNA amplification, diagnosis, molecular markers.

Introduction

In recent years, a great deal of information has been accumulated on the molecular alterations that take place during the development of tumors, such as gene mutations or genomic rearrangements, highlighting the possibility of detecting tumor alterations in biologic fluids and, consequently, indicating the use of these markers as a valid noninvasive diagnostic approach.

A tumor that has been widely investigated with this approach is colorectal cancer, which is one of the most common forms of cancer worldwide, with a clinical outcome varying considerably according to the type of lesion and stage of disease at diagnosis [1–3]. An early diagnosis is fundamental to reduce morbidity and mortality because a high percentage of patients diagnosed in the early stages of disease comprises long-term survivors [4]. Moreover, the possibility of detecting premalignant lesions makes this tumor an ideal target for screening programs. However, although several screening methods are available, a high percentage of individuals does not participate in colorectal cancer screening programs. There are many reasons for this low compliance, such as a lack of knowledge of the benefits of available screening methods, especially colonoscopy, as well as the unpleasant and troublesome procedures [5].

Gene mutations in stool, especially *K-ras* [6–12] and, to a lesser extent, *p53* [13], *APC* gene [14,15], and microsatellite instability [16], have been repeatedly investigated. Results have shown the presence of these molecular alterations in stool in only a fraction of patients, due to the relatively low frequency of single marker alterations in colorectal cancer. Multiple mutations have been analyzed in parallel on the same stool sample, and this approach has led to improved test sensitivity, but is expensive, time-consuming, and cannot easily be applied to screening programs [17–21].

The diagnostic potential of DNA amplification of exfoliated cells in stool has recently been considered. Preliminary evidence [19–21] has shown that the semiquantitative evaluation of DNA amplification (long DNA, or L-DNA) of some DNA fragments longer than 200 bp detects more than 50% of colorectal cancers, with a very high specificity.

In the present study, we aimed to discuss the results obtained from this inexpensive and rapid approach, both quantitative and objective, to increase its accuracy and thus permit a better discrimination between affected and nonaffected

Abbreviations: L-DNA, long DNA; FL-DNA, fluorescence long DNA; APC, adenomatous polyposis coli; ROC, receiver operating characteristic; FOBT, fecal occult blood test

Address all correspondence to: Daniele Calistri, PhD, Molecular Biology Laboratory, Division of Oncology and Diagnostics, Pierantoni Hospital, *Via* Forlanini 34, Forlì 47100, Italy. E-mail: biomolec@ausl.fo.it

[1]This work was supported by the Istituto Oncologico Romagnolo (Forlì, Italy) and the National Research Council (CNR; Roma, Italy).

Received 19 February 2004; Revised 23 April 2004; Accepted 28 April 2004.

DOI 10.1593/neo.04190

Individuals. For this purpose, we assessed the diagnostic potential of a new DNA amplification method (fluorescence long DNA, or FL-DNA) on a series of patients and healthy donors.

Patients and Methods

Case Series

Stool samples from 86 patients with primary colorectal cancer were collected in the Gastroenterology Unit and Department of Surgery I, Morgagni Hospital (Forlì, Italy) and in the Departments of Oncology and General Surgery, Infermi Hospital (Rimini, Italy). Stool samples were collected from 62 individuals who proved negative for cancer or benign lesions after colonoscopy, and from laboratory personnel.

Stool samples were obtained at least 3 days after the administration of laxative treatments in preparation for colonoscopy to allow for the recovery of normal bowel functionality. The fecal specimens were immediately frozen and stored at −70°C for a maximum of 2 months.

Cancer diagnosis was histologically confirmed and pathological stage was defined according to Dukes classification: 8 tumors were classified as stage A, 30 as stage B, 37 as stage C, and 9 as stage D. Moreover, 19 cancers were located in ascending colon, 30 in descending colon, 2 in transverse colon, and 35 in the rectal tract. Staging information was not available for only two cases.

Of the 86 patients, 42 were male and 44 were female, and median age was 72 years (range 36–90 years). Of the 62 controls, 29 were male and 33 were female, and median age was 51 years (range 21–87 years).

DNA Purification

Approximately 4 g of stool was thawed at room temperature. DNA was extracted after a 15-minute homogenization with 16 ml of TE-9 buffer pH 9 (0.5 M Tris–HCl, 20 mM EDTA, and 10 mM NaCl) by ULTRA-Turrax T25 (Janke and Kunkel GmbH and Co. KG IKA-Labortechnik, Staufen, Germany). After centrifugation at 5000*g* for 15 minutes, the supernatant was transferred to a tube containing 5 ml of 7.5 M ammonium acetate (M-Medical, Florence, Italy) and 30 ml of 100% ethanol (Carlo Erba, Milan, Italy). DNA was recovered by centrifugation at 5000*g* for 15 minutes at room temperature. Stool samples were suspended in 1.6 ml of ASL buffer and DNA was extracted using the QIAamp DNA Stool Kit (QIAGEN, Hilden, Germany).

L-DNA Analysis

p53 exons 5 to 8 and fragments 1 to 4 of *APC* exon 15 were amplified in a final volume of 25 μl containing 2 μl of DNA from stool, 0.4 μM of each primer, 200 μM deoxynucleotide triphosphates, 1 × reaction buffer with 3.5 mM $MgCl_2$, and 1 U of Taq polymerase (QIAGEN). The reaction mixture was subjected to 36 polymerase chain reaction (PCR) cycles: 60 seconds at 94°C, 60 seconds at 58°C, and 60 seconds at 72°C. Primer sequences without fluorescence-labeled 5′ ends have been described previously. Gel electrophoresis was performed by running 5 μl of PCR reaction in 2% agarose gel. In parallel, 5 μl of *K-ras* PCR product and a control plasmid were used to verify the presence of Taq inhibitors in fecal DNA samples. The relative amplification intensities of *p53* exons 5 to 8 and fragments 1 to 4 of APC were analyzed independently by two operators and classified as high, medium, low, or not detectable. An interobserver concordance was observed in all cases.

This semiquantitative L-DNA analysis was performed in parallel with the quantitative FL-DNA approach in 94 individuals (56 patients and 38 healthy volunteers) recruited during the first period of the study.

FL-DNA Analysis

Amplifications of exons 5 to 8 of *p53* and fragments 1 to 4 of *APC* exon 15 were carried out on 2 μl of DNA from stool in a total volume of 25 μl containing 0.4 μM of each primer, 200 μM deoxynucleotide triphosphates, 1 × reaction buffer with 3.5 mM $MgCl_2$, and 1 U of Taq polymerase (QIAGEN). The reaction mixture was subjected to 32 cycles: 60 seconds at 94°C and then 60 seconds at 60°C for *p53* exons, and 58°C for *APC* fragments, followed by incubation at 72°C for 60 seconds.

The *p53* exons were amplified simultaneously in a single reaction mixture and the four *APC* fragments were amplified in two different mixes (mix 1: fragments 1 and 2; mix 2: fragments 3 and 4). For this purpose, primers used for L-DNA analysis and those previously described [21] were end-labeled with fluorochromes provided by Applied Biosystems (Foster City, CA).

Electrophoresis was carried out using a 3100 Avant Genetic Analyzer (Applied Biosystems) equipped with GeneScan Analysis 3.7.

FL-DNA was performed by analyzing the fluorescence intensity of each sample-specific PCR product. The quantification of each sample was calculated by reference to a standard curve (1, 2, 5, 10, and 20 ng) of genomic DNA and expressed as nanograms. To verify the presence or absence of Taq inhibitors, amplification was performed on all samples with a mix containing 2 μl of DNA extracted from stool and 25 ag of a plasmid with a control sequence. All determinations were performed in duplicate and repeated in about 20% of samples in which the variation was >20%.

Statistical Analysis

FL-DNA concentrations were considered as a continuous variable. The most accurate cutoff values to discriminate between healthy donors and patients were calculated using the receiver operating characteristic (ROC) curve. In the ROC curves, sensitivity (true positive rate) was plotted against 1−specificity (false-positive rate) for all classification points.

Sensitivity, specificity, and relative 95% confidence interval (95% CI) were calculated for the most discriminant cutoff values.

Table 1. Sensitivity and Specificity of L-DNA Analysis.

High Amplifications	Healthy Donors	Patients	Sensitivity (%)	Specificity (%)
At least 1	8/38	39/56	70	79
≥2	1/38	28/56	50	97
≥3	0/38	20/56	36	100

Results

DNA amplification levels were evaluated by the semiquantitative L-DNA method on stool samples from a series of 56 patients with primary colorectal cancer and 38 healthy individuals. DNA levels were expressed as the number of high amplifications (i.e., as a discrete variable). A different distribution was observed in affected and unaffected individuals (Table 1). In particular, 79% (30 of 38) of healthy donors showed no amplification, whereas at least one amplification was observed in about 70% (39 of 56) of patients. The analysis of the accuracy of this approach using different cutoffs (Table 1) showed a very high specificity ranging from 79% to 100%. Conversely, sensitivity was very poor and did not exceed 70% at any cutoff value.

Amplification levels of fecal DNA were analyzed by the quantitative FL-DNA method in the overall series and expressed as a continuous variable (Figure 1). Only one stool sample from patients and three from healthy donors were not evaluable due to the presence of Taq inhibitors.

Fluorescence signals ranged from 0 to 283 ng (median, 47 ng) in patient stool and from 0 to 87 ng (median, 4 ng) in healthy donor stool. No differences in median values were observed with respect to age of patients and the size, site, and stage of tumor.

When the results from the two approaches were compared, a direct relation was observed, but with a wide variability of FL-DNA levels within the subgroups defined according to the number of L-DNA high amplifications. Moreover, fluorescence by FL-DNA method was detected in 33 of 47 individuals who did not show any high amplification by L-DNA assay. These results are clearly indicative of a higher sensitivity of the fluorescence method than of the conventional approach.

The ROC curve analysis of FL-DNA levels (Figure 2) shows a good diagnostic accuracy of this approach. In particular, very high specificity ranging from 83% to 95% and high sensitivity ranging from 82% to 72% were observed for the most discriminant cutoffs of 15, 20, 25, and 30 ng of DNA (Table 2). When the cutoff of 25 ng, which provides the best overall accuracy, was analyzed in relation to the different tumor characteristics, sensitivity remained high in patients with small tumors (70%) compared to large tumors

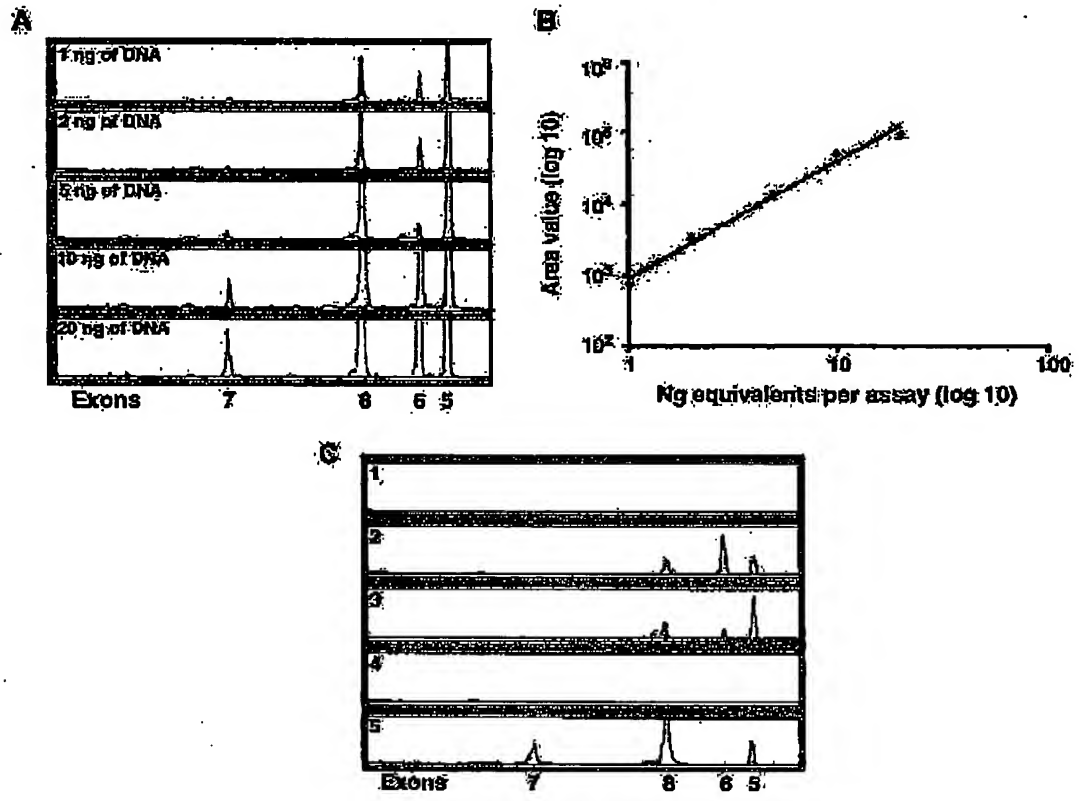


Figure 1. p53 analysis for FL-DNA quantification. (A) Amplification of scalar concentrations of genomic DNA using the same primers and conditions of stool sample determination. (B) The area values under the electropherogram peaks are plotted in a calibration curve. (C) p53 electropherograms of six stool samples. The amount of amplified DNA from individual samples is quantified on the basis of the calibration curve.

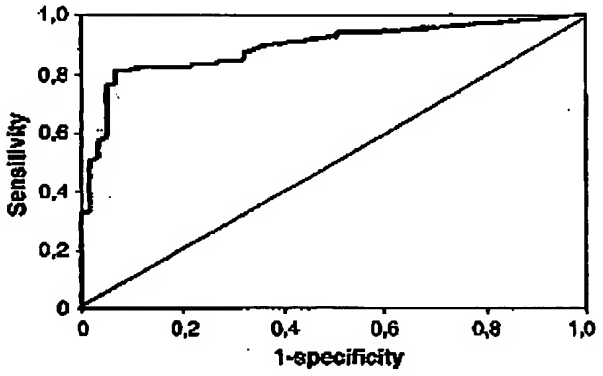


Figure 2. *ROC curve of FL-DNA analysis for the overall series of stool samples from patients and healthy donors.*

(82%) and was similar for the different Dukes stage tumors (Table 3). More importantly, a similar sensitivity was observed in detecting tumors localized in ascending and descending colon tracts.

Discussion

The possibility of performing population-based screening for colorectal cancer as well as for all tumor types is dependent on several factors such as complexity, time and cost, accessibility, and acceptability of screening methods.

Although several screening methods are currently available and have proven effective in reducing colorectal cancer mortality [22–27], a large-scale screening program comparable to those used for breast or prostate cancer does not exist. There are many reasons for this—the main one being the uncertainty of the best strategy to adopt [fecal occult blood test (FOBT), FOBT plus sigmoidoscopy, and so on]. Moreover, the real cost benefits of each method, considering the large number of endoscopic or radiologic procedures required for large-scale colorectal cancer screening, have not been determined [28–31]. Another important reason is that, for certain individuals, some of these techniques are not easily accepted, further reducing the compliance of the screening program itself [32].

A diagnostic approach that is less invasive, more accurate, and optimized in terms of time and cost is undoubtedly warranted. An important prospect is the analysis of molecular alterations detectable in human DNA extracted from stool. Many authors have investigated this area by analyzing a single molecular target or a combination of different molecular targets [6–21]. An interesting target that has recently been evaluated is the level of DNA amplification (L-DNA), which appears to be related to the presence and number of tumor cells in stool specimens. Only three studies to date have evaluated this marker in combination with some specific gene alterations using a semiquantitative method of analysis [19–21]. Their results show a good specificity and a relatively low sensitivity of this approach—the latter possibly due to the lack of an objective and quantitative evaluation, which compromises accurate discrimination between affected and nonaffected individuals, or to the insufficient sensitivity of the method.

In an attempt to improve the diagnostic accuracy of DNA amplification in exfoliated cells from stool, we set up and used an approach based on the evaluation of DNA amplification by a fluorescence method (FL-DNA). The results showed that this approach has a sensitivity comparable, if not superior, to that of multiple gene analysis, but is less expensive and less time-consuming. This sensitivity also made it possible to detect small, low-grade, and early-stage tumors. Moreover, unlike the fecal analysis of BAT26 instability or *K-ras* alterations, the determination of DNA amplification is able to detect tumors in all colon sites [16,33].

These unique features make this molecular marker an interesting tool for colorectal tumor diagnosis as it is characterized by all the benefits of other molecular analyses, such as noninvasiveness, simplicity, high compliance, reasonable costs, and time-efficient procedures.

Furthermore, this method could be improved and simplified by using alternative quantification systems such as chemoluminescence, spectrofluorimetry, real-time PCR, and so on, with the aim of developing kits that can be more easily utilized in all laboratories.

Table 3. Sensitivity* as a Function of Different Characteristics in Colorectal Cancer.

Category	Number of Patients	Positive	Negative	Sensitivity (%)
Size (cm)				
0.1–4.0	40	28	12	70
≥4.1	38	31	7	82
Dukes stage				
A	8	7	1	88
B	29	25	4	88
C	37	25	12	68
D	9	8	1	89
Location				
Ascending	18	13	5	72
Transverse	2	2	0	100
Descending	30	22	8	73
Rectum	35	26	7	80

*Cutoff value is 25 ng.

Table 2. Sensitivity and Specificity of FL-DNA Analysis.

DNA Levels Cutoff (ng)	Healthy Donors		Patients		Sensitivity (%)	95% CI	Specificity (%)	95% CI
	Positive	Negative	Positive	Negative				
15	10	49	70	15	82	(74–90)	83	(73–93)
20	7	52	70	15	82	(74–90)	88	(80–96)
25	4	55	66	20	76	(67–85)	93	(86–100)
30	3	56	61	24	72	(62–82)	95	(89–100)

A more exhaustive study, including adenomas and benign polyps, is needed to verify the real sensitivity and specificity of this method. However, these original preliminary results would seem to indicate the validity of this test and its potential usefulness in screening programs or in monitoring members of families at risk for colorectal cancer.

Acknowledgements

The authors thank Rosella Silvestrini for her invaluable scientific contribution; Gráinne Tierney for editing the manuscript; and the staff of the Gastroenterology Unit (Morgagni Hospital, Forlì) and Teresa Longhi (Department of General Surgery, Infermi Hospital, Rimini) for their assistance in collecting samples.

References

[1] Parkin DM, Whelan SL, Ferlay J, Raymond L, and Young J (Eds.) (1997). Cancer Incidence in Five Countries. IARC Scientific Publications No. 143 International Agency for Research on Cancer, Lyon.

[2] Boyle P (2002). Faecal occult blood testing (FOBT) as screening for colorectal cancer: the current controversy. *Ann Oncol* 13, 16–18.

[3] Strul H and Arber N (2002). Fecal occult blood test for colorectal cancer screening. *Ann Oncol* 13, 51–56.

[4] Ries LAG, Eisner MP, Kosary CL, Hankey BF, Miller BA, Clegg L, Mariotto A, Fay MP, Feuer EJ, and Edwards BK (Eds.) (2003). SEER Cancer Statistics Review, 1975–2000 National Cancer Institute. Bethesda, MD Available from http://seer.cancer.gov/csr/1975_2000/.

[5] Levin B, Brooks D, Smith RA, and Stone A (2003). Emerging technologies in screening for colorectal cancer: CT colonography, immunochemical fecal occult blood tests and stool screening using molecular markers. *CA Cancer J Clin* 53, 44–55.

[6] Sidransky D, Tokino T, Hamilton SR, Kinzler KW, Levin B, Frost P, and Vogelstein B (1992). Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. *Science* 256, 102–105.

[7] Hasegawa Y, Takeda S, Ichii S, Koizumi K, Maruyama M, Fujii A, Ohta H, Nakajima T, Okuda M, Baba S, et al. (1995). Detection of *K-ras* mutations in DNAs isolated from feces of patients with colorectal tumors by mutant-allele–specific amplification (MASA). *Oncogene* 10, 1441–1445.

[8] Smith-Ravin J, England J, Talbot C, and Bodmer W (1995). Detection of *c-Ki-ras* mutations in faecal samples from sporadic colorectal cancer patients. *Gut* 36, 81–86.

[9] Villa E, Dugani A, Rebecchi AM, Vignoli A, Grottola A, Buttafoco P, Losi L, Perini M, Tranda P, Morighi A, Larose R, and Manenti F (1996). Identification of subjects at risk for colorectal carcinoma through a test based on *K-ras* determination in the stool. *Gastroenterology* 110, 1346–1353.

[10] Nollau P, Moser C, Weinland G, and Wagener C (1996). Detection of *K-ras* mutations in stools of patients with colorectal cancer by mutant-enriched PCR. *Int J Cancer* 66, 332–336.

[11] Puig P, Urgell E, Capella G, Sancho FJ, Pujol J, Boadas J, Farré A, Lluis F, Gonzalez-Sastre F, and Mora J (2000). A highly sensitive method for *K-ras* mutation detection is useful in diagnosis of gastrointestinal cancer. *Int J Cancer* 85, 73–77.

[12] Prix L, Uciechowski P, Bockmann B, Giesing M, and Schuetz AJ (2002). Diagnostic biochip array for fast and sensitive detection of *K-ras* mutation in stool. *Clin Chem* 48, 428–435.

[13] Eguchi S, Kohara N, Komuta K, and Kanematsu T (1996). Mutations of the *p53* gene in the stool of patients with resectable colorectal cancer. *Cancer* 77, 1707–1710.

[14] Deuter R and Muller O (1998). Detection of *APC* mutation in stool DNA of patients with colorectal cancer by HD-PCR. *Human Mutat* 11, 84–89.

[15] Traverso G, Shuber A, Levin B, Johnson C, Olsson L, Schoetz DJ Jr, Hamilton SR, Boynton K, Kinzler KW, and Vogelstein B (2002). Detection of *APC* mutations in fecal DNA from patients with colorectal tumors. *N Engl J Med* 346, 311–320.

[16] Traverso G, Shuber A, Olsson L, Levin B, Johnson C, Hamilton SR, Boynton K, Kinzler KW, and Vogelstein B (2002). Detection of proximal colorectal cancer through analysis of faecal DNA. *Lancet* 359, 403–404.

[17] Renguccl C, Maiolo P, Saragoni L, Zoli W, Amadori D, and Calistri D (2001). Multiple detection of genetic alterations in tumors and stool. *Clin Cancer Res* 7, 590–593.

[18] Dong SM, Traverso G, Johnson C, Geng L, Favis R, Boynton K, Hibi K, Goodman SN, D'Alessio M, Paty P, Hamilton SR, Sidransky D, Baranv F, Levin B, Shuber A, Kinzler KW, Vogelstein B, and Jen J (2001). Detecting colorectal cancer in stool with the use of multiple genetic targets. *J Natl Cancer Inst* 93, 858–865.

[19] Ahlquist DA, Skoletsky JE, Boynton KA, Harrington JJ, Mahoney DW, Pierceall WE, Thibodeau SN, and Shuber AP (2000). Colorectal cancer screening by detection of altered human DNA in stool: feasibility of a multitarget assay panel. *Gastroenterology* 119, 1219–1227.

[20] Tagore KS, Lawson MJ, Yucaitis JA, Gage R, Orr T, Shuber AP, and Ross ME (2003). Sensitivity and specificity of a stool DNA multitarget assay panel for the detection of advanced colorectal neoplasia. *Clin Colorectal Cancer* 3, 47–53.

[21] Calistri D, Renguccl C, Bocchini R, Saragoni L, Zoli W, and Amadori D (2003). Fecal multiple molecular tests to detect colorectal cancer in stool. *Clin Gastroenterol Hepatol* 1, 377–383.

[22] Winawer SJ, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, Waye JD, Schapiro M, Bond JH, Panish JF, et al. (1993). Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup. *N Engl J Med* 329, 1977–1981.

[23] Muller AD and Sonnenberg A (1995). Prevention of colorectal cancer by flexible endoscopy and polypectomy. A case–control study of 32,702 veterans. *Ann Intern Med* 123, 904–910.

[24] Selby JV, Friedman GD, Quesenberry CP Jr, and Weiss NS (1992). A case–control study of screening sigmoidoscopy and mortality from colorectal cancer. *N Engl J Med* 326, 653–657.

[25] Hardcastle JD, Chamberlain JO, Robinson MH, Moss SM, Amar SS, Balfour TW, James PD, and Mangham CM (1996). Randomised controlled trial of faecal-occult-blood screening for colorectal cancer. *Lancet* 348, 1472–1477.

[26] Kronborg O, Fenger C, Olsen J, Jorgensen OD, and Sondergaard O (1996). Randomised study of screening for colorectal cancer with faecal-occult-blood test. *Lancet* 348, 1467–1471.

[27] Mandel JS, Bond JH, Church TR, Snover DC, Bradley GM, Schuman LM, and Ederer F (1993). Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. *N Engl J Med* 328, 1365–1371.

[28] Sonnenberg A, Delco F, and Inadomi JM (2000). Cost-effectiveness of colonoscopy in screening for colorectal cancer. *Ann Intern Med* 133, 573–584.

[29] Frazier AL, Colditz GA, Fuchs CS, and Kuntz KM (2000). Cost-effectiveness of screening for colorectal cancer in the general population. *JAMA* 284, 1954–1961.

[30] Lewis JD (2000). Prevention and treatment of colorectal cancer: pay now or pay later. *Ann Intern Med* 133, 647–649.

[31] Vijan S, Hwang EW, Hofer TP, and Hayward RA (2001). Which colon cancer screening test? A comparison of costs, effectiveness, and compliance. *Am J Med* 111, 593–601.

[32] Vernon SW (1997). Participation in colorectal cancer screening: a review. *J Natl Cancer Inst* 89, 1406–1422.

[33] Frattini M, Balestra D, Pilotti S, and Pierotti MA (2003). Tumor location and detection of *K-ras* mutations in stool from colorectal cancer patients. *J Natl Cancer Inst* 95, 72–79.

A Sensitive Method to Quantify Human Long DNA in Stool: Relevance to Colorectal Cancer Screening

Hongzhi Zou, Jonathan J. Harrington, Kristie K. Klatt, and David A. Ahlquist

Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, Minnesota

Abstract

Human long DNA in stool may reflect nonapoptotic exfoliation and has been used as a colorectal cancer (CRC) marker. Targeting human-specific *Alu* repeats represents a logical but untested approach. A real-time *Alu* PCR assay was developed for quantifying long human DNA in stool and evaluated in this study. The accuracy and reproducibility of this assay and the stability of long DNA during room temperature fecal storage were assessed using selected patient stools and stools added to human DNA. Thereafter, long DNA levels were determined in blinded fashion from 18 CRC patients and 20 colonoscopically normal controls. Reproducibility of real-time *Alu* PCR for quantifying fecal long DNA was high ($r^2 = 0.99$; $P < 0.01$). Long DNA levels in nonbuffered stools stored at room temperature fell a median of 75% by 1 day and 81% by 3 days. An EDTA buffer preserved DNA integrity during such storage. Human long DNA was quantifiable in all stools but was significantly higher in stools from CRC patients than from normal controls ($P < 0.05$). At a specificity of 100%, the sensitivity of long DNA for CRC was 44%. Results indicate that real-time *Alu* PCR is a simple method to sensitively quantify long human DNA in stool. This study shows that not all CRCs are associated with increased fecal levels of long DNA. Long DNA degrades with fecal storage, and measures to stabilize this analyte must be considered for optimal use of this marker. (Cancer Epidemiol Biomarkers Prev 2006;15(6):1115–9)

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the United States (1). Although CRC mortality is preventable if neoplasms can be detected at curable stage (2), only a minority of the population undergoes regular screening (3). Except for fecal occult blood testing, screening tools endorsed by the American Cancer Society are invasive and expensive (4).

The emergence of molecular stool testing provides a possible user-friendly alternative to conventional methods of CRC screening. A variety of DNA markers have been detected in the stools (5), including mutations of oncogenes (6) and tumor suppressor genes (7), microsatellite instability (8), and DNA methylation (9, 10). Owing to the continuous exfoliation of nonapoptotic neoplastic cells, long DNA occurs more abundantly in CRC stools than normal ones and serves as a candidate screening marker (11, 12). Colonocytes shed from normal epithelium undergo apoptosis, and their DNA is broken down by endonucleases into fragments shorter than 200 bp (12). However, there seems to be an escape from such apoptosis in exfoliated dysplastic cells, which results in long DNA sequences in stool that can be used for cancer detection (12).

Present methods for detecting long DNA use assay of multiple-specific target sequences on different genes (12, 13). Assay of *Alu* sequences represents a potentially simple approach to measure human long DNA in stool. *Alu* sequences embody the largest family of middle repetitive DNA sequences in the human genome. An estimated half million *Alu* copies are present per haploid human genome (14). Because *Alu* sequences are so abundantly distributed throughout the genome and specific to the genomes of primates (14), an assay that amplifies DNA sequences longer than 200 bp within these 300-bp repeats should provide a genome-wide approach to quantify human long DNA in stool. *Alu*-based assays have been used to quantify human tumor xenograft burden in murine (15) or chicken embryo models (16) as well as integrated HIV-1 DNA in infected HeLa cells (17) but have not been applied to stool.

This study was designed to (*a*) validate a real-time *Alu* PCR assay for quantifying human long DNA in stool, (*b*) evaluate the stability of long DNA in stool stored at room temperature and the effectiveness of an EDTA buffer for stabilizing DNA integrity, and (*c*) explore the feasibility of fecal long DNA quantification for CRC screening.

Materials and Methods

The study was approved by the Mayo Clinic Institutional Review Board.

Stool DNA Extraction. Total DNA was extracted from stool samples with QIAamp DNA Stool Mini kit (Qiagen, Valencia, CA). Stool (2 g) was homogenized in 20 mL buffer ASL, and stool slurry (2 mL) was then used to extract total DNA following the instruction of the manufacturer. DNA was finally eluted in 100 μL buffer AE.

Real-time *Alu* PCR. The *Alu* sequence consists of conserved regions and variable regions. In the putative consensus *Alu* sequence, the conserved regions are the 25-bp span between nucleotide positions 23 and 47 and 16-bp span between nucleotide positions 245 and 260 (14). Although primers may be designed in any part of the *Alu* sequences for more effectively amplifying *Alu* sequences, the PCR primers should completely or partially (at least the 3′-regions of the primers) locate in the conserved regions. Primers specific for the human *Alu* sequences [sense (5′-ACGCCTGTAATCCCAGCACTT-3′) and antisense (5′-TCGCCCAGGCTGGAGTGCA-3′)] were used to amplify sequences ~245 bp inside *Alu* repeats (Fig. 1; ref. 16). Stool DNA was diluted 1:5 with 1× Tris-EDTA buffer (pH 7.5) for PCR amplification. Tris-EDTA buffer–diluted stool DNA (1 μL) was amplified in a total volume of 25 μL containing 1× iQ SYBR Green Supermix

Received 12/28/05; revised 3/3/06; accepted 4/11/06.

Grant support: Charles Oswald Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: David A. Ahlquist, Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN 55905. Phone: 507-266-4338; Fax: 507-266-0350. E-mail: ahlquist.david@mayo.edu

doi:10.1158/1055-9965.EPI-05-0992

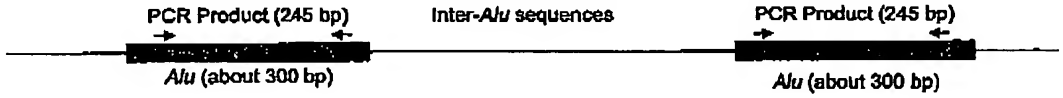


Figure 1. The design of the real-time *Alu* PCR. Primers with 3'-ends complementary to the conserved regions of consensus sequence were used to amplify products ~245 bp inside *Alu* repeats.

(Bio-Rad, Hercules, CA), 200 nmol/L each primer under the following conditions: 95°C for 3 minutes followed by 23 cycles of 95°C and 60°C for 30 seconds and 72°C for 40 seconds. Standard curve was created for each plate by amplifying 10-fold serially diluted human genomic DNA samples (Novagen, Madison, WI). Melting curve was made after each PCR to guarantee that only one product was amplified for all samples. Amplification was carried out in 96-well plates in an iCycler (Bio-Rad). Each plate consisted of stool DNA samples and multiple positive and negative controls. Each assay was done in duplicate.

Long DNA Stability Analysis. Five fresh stools from CRC patients were used to test the stability of human long DNA in stool stored at room temperature. Four aliquots (2 g each) from each of the five stools were stored at room temperature for 0, 1, 3, and 8 days. Total stool DNA was extracted from each aliquot with QIAamp DNA Stool Mini kit as described above. Human long DNA in total stool DNA sample was quantified with real-time *Alu* PCR as described above. Long DNA levels in stool aliquots extracted in days 1, 3, and 8 were divided by long DNA level in the stool aliquot extracted in day 0 for each stool sample to calculate the percentage of intact long DNA kept in the stool aliquots stored at room temperature for different durations. The median percentage of long DNA kept at each time point for five stools was then calculated.

Stabilizing Human DNA Integrity. Four fresh normal stools with added human genomic DNA were used to test the effectiveness of an EDTA-based buffer for stabilizing DNA integrity in stools. Human genomic DNA (1 μg) was spiked into two aliquots (4 g each) of each stool, and then aliquots of each stool were homogenized with 40 mL of two different buffers, including buffer with 100 mmol/L EDTA [0.5 mol/L Tris, 10 mmol/L NaCl, 100 mmol/L EDTA (pH 7); ref. 18] and buffer with 16 mmol/L EDTA [0.5 mol/L Tris, 10 mmol/L NaCl, 16 mmol/L EDTA (pH 7)]. Homogenized stool slurry was stored at room temperature, and 2 mL of it was used for stool DNA extraction at each of four different time points (day 0, 1, 3, and 8). Total stool DNA was extracted from each aliquot with QIAamp DNA Stool Mini kit with some modifications. Human DNA in total stool DNA sample was quantified with real-time *Alu* PCR. The median percentage of human DNA kept at each time point was calculated.

Clinical Pilot Study. A completely independent set of fresh stools from 18 CRC patients and from 20 colonoscopically normal individuals were analyzed in blinded fashion. The demographic and clinical characteristics of the CRC patients and controls are shown in Table 1. All stools were collected before colonoscopy or surgery. None of the CRC patients had undergone chemotherapy or radiotherapy before stool collection. Any previous instrumentation had occurred >2 weeks before stool collection. A plastic bucket device was used to collect whole stool. Stools in sealed buckets were immediately transported to our laboratory, and total DNA was extracted from all stools within 4 hours from defecation.

Statistical Analysis. For human long DNA levels obtained by real-time *Alu* PCR, the median for each group of stool samples was calculated, and Wilcoxon signed-rank test was used to compare the human long DNA levels of different stool groups. Spearman's rank correlation was used to calculate the correlation coefficient of the reproducibility. Statistical tests were done using SAS statistical software (SAS Institute, Inc., Cary, NC). All *P*s were two sided.

Table 1. Demographic and clinical characteristics of subjects

	Cancer	Normal
Number	18	20
Sex (M/F)	12/6	11/9
Mean age (y)	62	71
Site (proximal/distal)	5/13	
Median size, cm (range)	3.5 (1.1-10.0)	
Stage (Dukes AB/CD)	8/9*	

*Duke stage information was not available for a patient who did not have surgery.

Results

Validating Real-time *Alu* PCR Assay. To determine the dynamic range of the real-time *Alu* PCR, human genomic

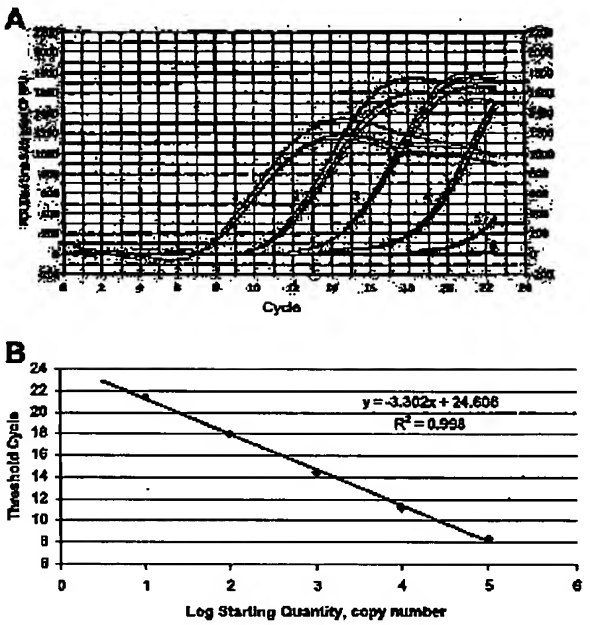


Figure 2. A. Human genomic DNA samples, which had been serially diluted by 10-fold (*lines 1*, 2.5 ng; *lines 2*, 250 pg; *lines 3*, 25 pg; *lines 4*, 2.5 pg; and *lines 5*, 250 fg), were amplified with real-time *Alu* PCR. Water control, 2.5 ng of each genomic DNA from pig, bovine, and chicken, and 2.5 ng *E. coli* genomic DNA were not amplified with real-time *Alu* PCR (*lines 6*). B. A standard curve was created with the log starting quantity and threshold cycle of the 10-fold serially diluted human genomic DNA samples.

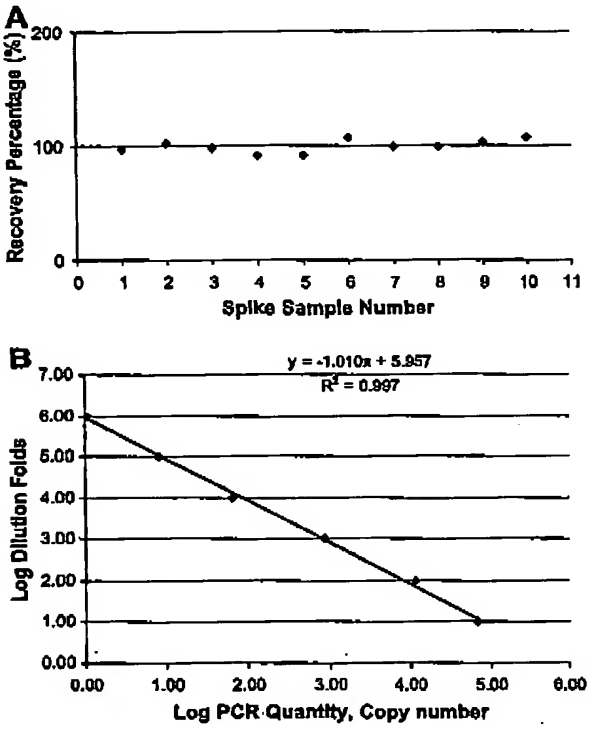


Figure 3. A. Human genomic DNA (25 pg) added into 10 different stool DNA samples was recovered by real-time *Alu* PCR. Recovery percentage (%) equals to human DNA amount recovered divided by human DNA amount added and then multiplied by 100. B. A stool DNA sample was 10-fold serially diluted, and human long DNA was then quantified using real-time *Alu* PCR. Linear recovery of human long DNA in serially diluted stool DNA samples.

DNA samples serially diluted over a 10-fold range (2.5 ng, 250 pg, 25 pg, 2.5 pg, 250 fg, and 25 fg) were amplified with the real-time *Alu* PCR. *Alu* sequences were linearly detected from 250 fg up to 2.5 ng human genomic DNA per PCR (Fig. 2A and B).

To confirm the human specificity of the real-time *Alu* PCR, genomic DNA samples from pig, bovine, and chicken, which are three nonhuman species typically consumed in the diet, and *Escherichia coli*, a common bacterium in stool, were tested by this method. The *Alu*-based PCR assay was negative for all nonhuman mammalian DNA and *E. coli* DNA (Fig. 2A).

Because stool contains PCR inhibitors (19), quantification could be affected by PCR inhibitors. To check whether assay accuracy was affected by potential PCR inhibitors, 500 pg human genomic DNA (2 μL) was added into 10 different stool DNA samples (38 μL each), and mixed DNA (1 μL), which contained 25 pg human genomic DNA, was then quantified with real-time *Alu* PCR. The mean recovery percentage of the added samples was 99.6% (range, 91.4-107.8%; Fig. 3A). For further confirming that PCR inhibitors did not affect the quantitative accuracy of the assay, one stool DNA sample from a CRC patient was 10-fold serially diluted and then quantified with real-time *Alu* PCR. Linear recovery of long DNA from these serially diluted stool DNA aliquots ($r^2 = 0.997$) confirmed the absence of interference by PCR inhibitors (Fig. 3B).

The reproducibility of the real-time *Alu* PCR was studied in frozen stool samples from eight CRC patients and eight normal individuals. Human long DNA in these stool DNA samples was quantified in duplicate. The human long DNA levels of duplicate runs correlated highly ($r^2 = 0.99$; $P < 0.01$; Fig. 4).

Instability of Human Long DNA in Stools Stored at Room Temperature. Compared with stools tested on day 0, median long DNA levels in stools stored at room temperature for 1, 3, and 8 days after defecation fell 75%, 81%, and 89%, respectively (Fig. 5A).

From four fresh normal stools added to human DNA and mixed with low concentration EDTA (16 mmol/L), recoveries of human DNA after room temperature storage for 1, 3, and 8 days were 65%, 19%, and 3%, respectively, compared with day 0. However, for stool aliquots mixed in buffer with high EDTA concentration (100 mmol/L), median recoveries of added human DNA were preserved at 121%, 118%, and 100%, respectively (Fig. 5B).

Human Long DNA Levels in CRC Stools and Normal Controls. Human long DNA levels in 18 CRC and 20 normal fresh stools, which were collected immediately after defecation, were quantified by real-time *Alu* PCR in blinded fashion. Human long DNA was detected in all 38 stool samples but was significantly higher in CRC stools (median, 309 ng/g stool; range, 5-21,115) than in normal stools (median, 70 ng/g stool; range, 2-2,870; $P = 0.04$; Fig. 6). At a long DNA cutoff of 2,900 ng/g stool, sensitivity for CRC was 44% (8/18), and specificity was 100% (20/20). Median long DNA in five proximal CRC stools was 48 ng/g (range, 10-506 ng/g) and in 13 distal CRC stools was 4264 ng/g

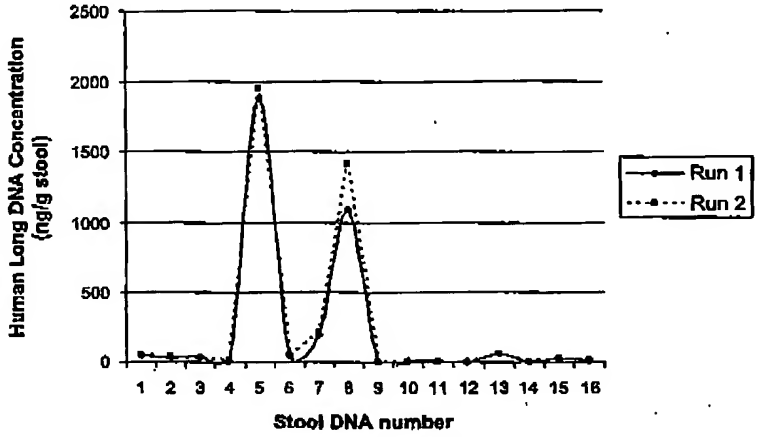


Figure 4. Stool DNA samples from eight CRC and eight normal stools were quantified with real-time *Alu* PCR twice. The human long DNA levels from these two runs showed good reproducibility ($r^2 = 0.99$).

(range, 5-21,115; $P = 0.09$). In this small series, tumor size did not significantly affect long DNA levels in stool.

Discussion

This report describes a new method to quantify human long DNA in stool using real-time PCR amplification of a 245-bp sequence within *Alu* repeats. The method is very sensitive with a dynamic range of 250 fg to 2.5 ng human genomic DNA, accurately detects human DNA added into stools, and yields highly reproducible results. Furthermore, this real-time *Alu* PCR method may have advantages of simplicity and speed compared with other approaches that describe use of multiple gene targets to assay long human DNA in stool (12, 13).

With this validated new method, we found that human long DNA was present in all stools tested, but levels were significantly higher in stools from CRC patients than from normal individuals. When human long DNA in stool was used as a marker at a 100% specificity cutoff, about half of CRC patients could be detected, which is consistent with the performance of long DNA as a marker in earlier reports (11-13). The abundance of human long DNA in stools from CRC patients likely reflects the nonapoptotic exfoliation that occurs with CRC described by others (11-13).

In two recent multicenter studies (20, 21), human long DNA in stool was less informative than in earlier reports. This discrepancy seems to be due to degradation by bacterial DNAases during prolonged preassay fecal storage that occurred with mailed-in samples in these studies. Experimental observations in the present study and by others (18) corroborate the instability of human long DNA during fecal storage. Such degradation can be prevented by mixing stools with buffers containing a DNAase inhibitor like EDTA (18) as was shown in the present study. If human long DNA is to be used clinically as a fecal marker, then attention must be given to incorporating a DNAase inhibitor as part of specimen collection and processing.

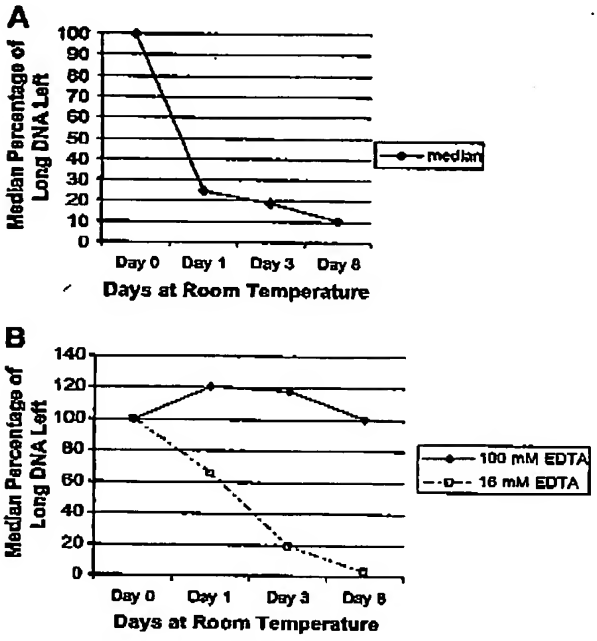


Figure 5. A. Long DNA levels of CRC stools stored at room temperature for 1, 3, and 8 days after defecation fell median percentages of 75%, 81%, and 89%, respectively. B. Buffers with 100 mmol/L EDTA and 16 mmol/L EDTA showed different effects on preserving human DNA added in stools stored at room temperature.

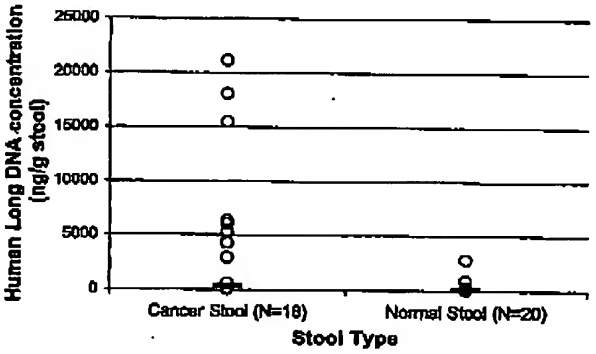


Figure 6. Human long DNA levels of stools in the blinded pilot clinical study. O, stool sample. *Solid horizontal bar*, median of human long DNA concentration within a group of subjects.

Human long DNA is not specific for CRC. Preliminary reports suggest that human long DNA in stool may detect cancers in the upper gastrointestinal track as well (22). Inflammatory bowel disease has also been shown to be associated with elevated levels of human long DNA in stools (23). In contrast to normal epithelial cells, which undergo apoptosis (anoikis) when shed from their basement membrane attachment (24), inflammatory cells are anchorage independent and logically contribute to long DNA in stools. The discriminant value of human long DNA measured by this method would need to be verified in a larger and more representative sample if it were to be considered for screening or other clinical applications.

Real-time *Alu* PCR is a simple, rapid, and inexpensive method for quantifying human long DNA in stools. This method may have useful applications for research observations and clinical testing.

Acknowledgments

We thank Tammy S. Neseth and Ann Kolb for collecting samples.

References

1. Jemal A, Murray T, Ward E, et al. Cancer statistics 2005. CA Cancer J Clin 2005;55:10–30.
2. Mandel JS, Bond JH, Church TR, et al. Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. N Engl J Med 1993;328:1365–71.
3. From the centers for disease control and prevention. Colorectal cancer test use among persons aged > or = 50 years—United States, 2001. JAMA 2003; 289:2492–3.
4. Levin B, Brooks D, Smith RA, Stone A. Emerging technologies in screening for colorectal cancer: CT colonography, immunochemical fecal occult blood tests, and stool screening using molecular markers. CA Cancer J Clin 2003; 53:44–55.
5. Osborn NK, Ahlquist DA. Stool screening for colorectal cancer: molecular approaches. Gastroenterology 2005;128:192–206.
6. Sidransky D, Tokino T, Hamilton SR, et al. Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. Science 1992;256:102–5.
7. Traverso G, Shuber A, Levin B, et al. Detection of APC mutations in fecal DNA from patients with colorectal tumors. N Engl J Med 2002;346:311–20.
8. Traverso G, Shuber A, Olsson L, et al. Detection of proximal colorectal cancers through analysis of faecal DNA. Lancet 2002;359:403–4.
9. Ahlquist DA, Klatt KK, Harrington JJ, Cunningham JM. Novel use of hypermethylated DNA markers in stool for detection of colorectal cancer: a feasibility study. Gastroenterology 2002;122 Suppl:A40.
10. Muller HM, Oberwalder M, Fiegl H, et al. Methylation changes in faecal DNA: a marker for colorectal cancer screening? Lancet 2004;363:1283–5.
11. Ahlquist DA, Skoletsky JE, Boynton KA, et al. Colorectal cancer screening by detection of altered human DNA in stool: feasibility of a multitarget assay panel. Gastroenterology 2000;119:1219–27.

12. Boynton KA, Summerhayes IC, Ahlquist DA, Shuber AP. DNA integrity as a potential marker for stool-based detection of colorectal cancer. Clin Chem 2003;49:1058–65.
13. Calistri D, Rengucci C, Bocchini R, Saragoni L, Zoli W, Amadori D. Fecal multiple molecular tests to detect colorectal cancer in stool. Clin Gastroenterol Hepatol 2003;1:377–83.
14. Kariya Y, Kato K, Hayashizaki Y, Himeno S, Tarui S, Matsubara K. Revision of consensus sequence of human Alu repeats—a review. Gene 1987;53:1–10.
15. Schneider T, Osl F, Friess T, Stockinger H, Scheuer WV. Quantification of human Alu sequences by real-time PCR—an improved method to measure therapeutic efficacy of anti-metastatic drugs in human xenotransplants. Clin Exp Metastasis 2002;19:571–82.
16. Zijlstra A, Mellor R, Panzarella G, et al. A quantitative analysis of rate-limiting steps in the metastatic cascade using human-specific real-time polymerase chain reaction. Cancer Res 2002;62:7083–92.
17. Brussel A, Sonigo P. Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. J Virol 2003;77:10119–24.
18. Olson J, Whitney DH, Durkee K, Shuber AP. DNA stabilization is critical for maximizing performance of fecal DNA-based colorectal cancer tests. Diagn Mol Pathol 2005;14:183–91.
19. Deuter R, Pietsch S, Hertel S, Muller O. A method for preparation of fecal DNA suitable for PCR. Nucleic Acids Res 1995;23:3800–1.
20. Imperiale TF, Ransohoff DF, Itzkowitz SH, Turnbull BA, Ross ME. Colorectal Cancer Study Group. Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. N Engl J Med 2004;351:2704–14.
21. Ahlquist DA, Sargent DJ, Levin TR, et al. Stool DNA screening for colorectal cancer: prospective multicenter comparison with hemoccult. Gastroenterology 2005;128:63.
22. Ahlquist D, Cameron A, Jett J, Pearson R. Universal detection of aerodigestive cancers by assay of nonapoptotic human DNA in stool. Gastroenterology 2002;118 Suppl:A855.
23. Itzkowitz S, Jandorf L, Ullman T, et al. Stool DNA testing identifies patients with inflammatory bowel disease and dysplasia. Gastroenterology 2005;128: A567.
24. Shanmugathasan M, Jothy S. Apoptosis, anoikis, and their relevance to the pathobiology of colon cancer. Pathol Int 2000;50:273–9.

Gastroenterology
Official Journal of the AGA Institute

GASTROENTEROLOGY HOME
CURRENT ISSUE
ARTICLES IN PRESS
PREVIOUS ISSUES
ARTICLE COLLECTIONS
• Gastroenterology and Hepatology News
• Comments from the Editors
• Clinical Challenges and Images in GI
• Imaging and Advanced Technology
• Reviews in Basic and Clinical Gastroenterology
• AGA Institute Position Papers
• Selected Summaries
• Print And Media Reviews
• Correspondence
ONLINE CME
SEARCH GASTROENTEROLOGY
CONTACT US
JOURNAL INFORMATION
• About Gastroenterology
• Article Statistics
• Board of Editors
• Editorial Board
• Editoral Staff
• Pricing
• Email Notification
• Info for Advertisers
• Press Embargo
INFO FOR AUTHORS
• Submit a Manuscript
• Instructions for Authors
• Abstracting/Indexing
• Copyright Form
• CONSORT Checklist
• Permission to Reuse
• Style Guide
RESOURCES
JOIN AGA
SHARED SCIENCE
SUBSCRIBE

Volume 119, Issue 5,
Pages 1219-1227 (01 November 2000)

previous 4 of 23 next

Colorectal Cancer Screening by Detection of Altered Human DNA in Stool: Feasibility of a Multitarget Assay Panel☆☆☆☆☆☆☆

David A. Ahlquist*, Joel E. Skoletsky†, Kevin A. Boynton†, Jonathan J. Harrington*, Douglas W. Mahoney*, William E. Pierceall†, Stephen N. Thibodeau*, Anthony P. Shubert

ABSTRACT
FULL-TEXT PDF (242 KB)
CITATION ALERT
CITED BY
RELATED ARTICLES
EXPORT CITATION
EMAIL TO A COLLEAGUE

Abstract

Background & Aims: Assay of altered DNA exfoliated into stool represents an intriguing approach to screen for colorectal neoplasia, but multiple markers must be targeted because of genetic heterogeneity. We explored the feasibility of a stool assay panel of selected DNA alterations in discriminating subjects with colorectal neoplasia from those without. **Methods:** Freezer-archived stools were analyzed in blinded fashion from 22 patients with colorectal cancer, 11 with adenomas ≥1 cm, and 28 with endoscopically normal colons. After isolation of human DNA from stool by sequence-specific hybrid capture, assay targets included point mutations at any of 15 sites on K-*ras*,*p53* , and *APC* genes; Bat-26, a microsatellite instability marker; and highly amplifiable DNA. **Results:** Analyzable human DNA was recovered from all stools. Sensitivity was 91% (95% confidence interval, 71%–99%) for cancer and 82% (48%–98%) for adenomas ≥1 cm with a specificity of 93% (76%–99%). Excluding K-*ras* from the panel, sensitivities for cancer were unchanged but decreased slightly for adenomas to 73% (39%–94%), while specificity increased to 100% (88%–100%). **Conclusions:** Assay of altered DNA holds promise as a stool screening approach for colorectal neoplasia. Larger clinical investigations are indicated.

No full text is available. To read the body of this article, please view the PDF online.

*Division of Gastroenterology and Hepatology, Department of Biostatistics, and Division of Molecular Genetics, Mayo Clinic, Rochester, Minnesota; and †Applied Research Group, EXACT Laboratories, Maynard, Massachusetts

☆ Supported by grants from the National Cancer Institute of National Institutes of Health (R01 CA 7160), EXACT Laboratories (Maynard, MA), and Mayo Foundation (Rochester, MN) and by the philanthropic support of Charles W.

Oswald.

*** The authors thank Drs. Bert Vogelstein and Kenneth W. Kinzler of Johns Hopkins University Oncology Center and Howard Hughes Medical Institute for helpful comments through the course of this study; Joy Yucaitis for extensive logistical support; Jaci McCormick, Mary Devens, Joel Swenson, and Nancy Diehl for valuable database support; Dr. Lawrence Burgart for pathology review; and Drs. Steven J. Laken and Barry Berger for contributions to technical aspects of assay interpretation and critical scientific input. The authors also thank the following individuals for expertise and dedicated efforts: Kristina Stuhr Brennan (sample preparation); Cynthia LeSage Proulx, Jean A. Smith, J. Kent Moore, David Morrissey, and Sharon Magnolo (DNA purifications and amplifications); Pamela Shaw and Beverly M. Vucson (mutation detection and analyses); and Lauren A. Robison (quality assurance/quality control support).

* Mayo Foundation is a minor equity investor in EXACT Laboratories. D. Ahlquist is a member of EXACT Laboratories' Scientific Advisory Board but holds no stock and has received no consulting fees. J. Harrington and D. Mahoney have no affiliation with EXACT Laboratories. A. Shuber, J. Skoletsky, W. Pierceall, and K. Boynton are employees of EXACT Laboratories.

** Presented in part at the annual meeting of the American Gastroenterological Association in Orlando, Florida, in May 1999 (Gastroenterology 1999;116:A369).

PII: S0016-5085(00)89526-8

doi:10.1053/gast.2000.19580

method for colorectal tumor detection based on quantitative analysis of DNA from a stool sample.

None of the publications of TIAN, KMIEC, ALBERTSEN, or BUCK disclose or suggest a method for detecting the presence of colorectal tumors, wherein the total amount of amplicons higher than the reference value is indicative of the presence of a colorectal tumor in a subject.

Thus, in view of the above, applicants respectfully request that the obviousness rejections be withdrawn.

At this time, applicants respectfully request an action on the merits for all of the claims in their full scope. Applicants maintain that the lack of unity determination is improper for the same reasons outline in the response of January 22, 2007 and again ask that the Examiner withdraw the requirement.

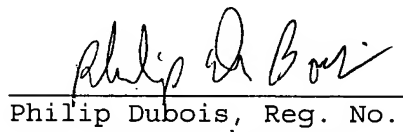
In view of the present amendment and foregoing remarks, therefore, applicants believe that the present application is in condition for allowance at the time of the next Official action. Allowance and passage to issue on that basis is respectfully requested.

Please charge the fee of $25 for one extra dependent claim added herewith to Deposit Account No. 25-0120.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

YOUNG & THOMPSON



Philip Dubois, Reg. No. 50,696
745 South 23rd Street
Arlington, VA 22202
Telephone (703) 521-2297
Telefax (703) 685-0573
(703) 979-4709

PD/lrs